Quantitative Evaluation of the Antifungal Properties of Cycloheximide

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Although the total growth of both zoopathogenic and saprophytic fungi on cycloheximide media was consistently less than that on the control without the antibiotic, a progressive increase in the growth rate of these organisms occurred during exposure to the drug. The extent of this change depended upon the concentration of cycloheximide, the species and strain of the test organism, and the duration of exposure. Significant alterations were also observed in the macroscopic appearance of the colonies. The results of this investigation agree with those of previous studies regarding the value of cycloheximide in selective isolation media, but there were discrepancies with respect to the degree of sensitivity of several of the organisms studied. The increase in the rate of growth on cycloheximide media may indicate an induced adaptation to the drug.

Cycloheximide is a glutarimide derivative originally isolated in crystalline form by Leach et al. (7) from certain streptomycin-yielding strains of Streptomyces griseus. Whiffen (16, 17), the first to explore the significant antifungal properties of this antibiotic, found that zoopathogenic fungi were 10 to 15 times more resistant to the antibiotic than phytopathogens or saprophytes. Subsequently, Georg et al. (4, 5) studied the potential use of cycloheximide in the selective isolation of dermatophytes. They reported that the addition of the antibiotic to Sabouraud dextrose agar (SDA) greatly facilitated the isolation of zoopathogens by curtailing the development of contaminating saprophytes. Since these early studies, recently reviewed by Sisler and Siegel (12) and Tsao (15), numerous reports have been published concerning the antifungal spectrum of cycloheximide, its mechanism of action, and its use in a variety of isolation media. However, many of these investigations of its antifungal properties suffer from the same major deficiencies. First, they have been primarily qualitative studies in which the data were generally obtained from subjective macroscopic observations of the appearance of the cultures, and then usually reported in terms of a numerical scale of 0 to 4. Second, with minor exceptions, these reports considered only 1 concentration, or at the most three concentrations, of cycloheximide. Third, the results were generally derived from one or two observations during an extremely short experimental period of only 3 to 10 days.

The increase in the reported prevalence of fungal diseases (9–11) and the recent development of "simplified" isolation media (1, 13, 14) have necessitated a reevaluation of the antifungal properties of this antibiotic. Therefore, in this report, we present data from a quantitative analysis of the activity of cycloheximide with respect to both zoopathogenic and saprophytic fungi.

MATERIALS AND METHODS

Test organisms. The two strains of each of the five genera employed in this study were divided into two experimental sets. The first of these consisted of Trichophyton rubrum strain 689, Histoplasma capsulatum strain 219A, Allescheria boydii strain 1, Cladosporium sp. strain 242A, and Scopulariopsis sp. strain 326. The second set was composed of T. rubrum strain 379, H. capsulatum strain 211, A. boydii strain 17A, C. cladosporioides sp. strain 242, and S. scopulariopsis sp. strain 61-12. Axenic cultures of these organisms, obtained from the collection of the Laboratories for Mycology in our institution, were maintained at 27°C on standard SDA (2% dextrose, 1% Neopeptone, and 2% agar).

Media. The five concentrations of cycloheximide studied in these experiments (0.5, 1.0, 0.5, 0.1, and 0.05 mg/ml) were prepared by dissolving the crystalline form of the antibiotic (Actidione, The Upjohn Co., Kalamazoo, Mich.) in 0.067 M solutions of KH₂PO₄, as described by Georg et al. (5). These solu-

1 A preliminary report of this work was presented at the 5th Congress of the International Society for Human and Animal Mycology, Paris, France, 5–10 July 1971.
tions were then sterilized by passage through 0.45-μm Nalgene filter units (Nalge Sybron Corp., Rochester, N.Y.) and added to molten, sterilized SDA. The SDA-cycloheximide media were dispensed by means of sterile Salvarsan tubes (Kimble Glass, Toledo, Ohio) into 100-mm glass petri dishes, 30 ml/plate, with triplicate plates prepared for each concentration of antibiotic. SDA without cycloheximide served as a control.

Inoculation. By means of a stainless-steel cork borer, 4-mm inoculum plugs were removed from the edge of 2-week-old stock cultures and transferred to precut wells of the same size in the center of the experimental plates. All test cultures were incubated at 27°C.

Determination of colonial growth. At 2-day intervals, the diameter of the mycelial mat was measured under an 8-power dissecting microscope along two mutually perpendicular axes marked on the bottom of the plate. The average diameter was computed from triplicate plates and corrected for the 4 mm of the inoculum plug. The measurements for each concentration of cycloheximide and for the control were compared, and from these data growth relative to the control was calculated.

Since A. boydii and Scopulariopsis sp. on the control medium reached the edge of the petri dish 17 days after inoculation, for comparative purposes all experiments were terminated at that time.

Colonial morphology. To determine the effects of cycloheximide on the gross morphology of the colonies, macroscopic observations were made of all cultures at the completion of the experiments.

RESULTS

Although total growth of each of the test organisms on cycloheximide media was less than that on SDA alone, data from duplicate tests with each experimental set revealed an increase in the rate of growth of most species as compared with that on the control medium during exposure to the antibiotic. The presence and extent of this alteration depended upon the species and strain of microorganism and the concentration of antibiotic.

The amount of growth of both strains of T. rubrum on test media relative to that on the control (Fig. 1 and 2) remained fairly constant at all levels of the antibiotic below 5.0 mg/ml, at which concentration an increase with time was observed. Per cent growth of H. capsulatum 211 either remained constant or decreased slightly at levels of cycloheximide below 5.0 mg/ml, whereas that of H. capsulatum 219A increased. Per cent growth of both strains of A. boydii increased

![Graph](image-url)
with time at all concentrations of cycloheximide, with the degree of change for strain 1 inversely related to the level of antibiotic. The magnitude of the increase with both strains was, in general, greater than that noted with other zoopathogens.

Although a considerable quantitative difference in response was noted between the two strains of *Cladosporium*, both displayed a marked increase in growth, the extent of which varied directly with the concentration of cycloheximide and was greater than with any other test organism. The increase in per cent growth noted with both strains of *Scopulariopsis* was inversely related to the concentration of antibiotic.

The increase in percentage of growth was a measure of the change in response of the organisms to cycloheximide during exposure to the antibiotic (Fig. 1 and 2). On the third day after inoculation, growth of *Cladosporium* relative to the control only slightly exceeded that of *Scopulariopsis*. Development of both organisms was sharply curtailed, rarely exceeding 40% of that on the control. Development of the zoopathogens at this time was greatly restricted only at 5.0 mg/ml; at lower levels, the growth of *T. rubrum* and *H. capsulatum* was 75 to 90% of that on the control. Although the percentage of growth of *A. boydii* was lower than that of the other zoopathogens (about 50%), it was still greater than that of either saprophyte.

In contrast, by the 17th day after inoculation, a different pattern of responses had emerged. First, growth of *Cladosporium* strain 242A relative to its development on the control was greater than that observed with the zoopathogens at 5.0 mg/ml, and the percentage of growth of strain 242, at all concentrations, exceeded that of both strains of *A. boydii*, of *H. capsulatum* 211, and of *T. rubrum* 689. Second, relative growth of *A. boydii* strain 1 approximated or exceeded that of both strains of *T. rubrum* and of *H. capsulatum* 211 at all concentrations except 5.0 mg/ml. Third, per cent growth of *H. capsulatum* 219A, at all levels but 5.0 mg/ml, was greater than that found with any other test organism.

Exposure to cycloheximide for 17 days resulted in alterations in colonial morphology of several of the test organisms. Such a change in the appearance of both strains of *T. rubrum* was noted at 5.0 mg of antibiotic/ml (Fig. 3 and 4), the mycelial mat being closely appressed to, or embedded in, the agar and consisting of finely
interwoven hyphae that gave the colony a hyaline appearance. On the control medium, the mycelial mat was downy and slightly mounded on the surface of the agar.

Whereas cycloheximide had no apparent effect upon the morphology of *H. capsulatum* 219A, significant changes occurred in *H. capsulatum* 211. On control medium (Fig. 5F), the mycelial mat was flat and generally downy in texture, except at the center (inoculation point). In the latter area, the colony was cerebriform and granular, the granules being composed of densely packed interwoven hyphae. At 5.0 mg of antibiotic/ml (Fig. 5A), the colony lacked granules and was mounded on the surface of the agar. At the remaining concentrations of cycloheximide (Fig. 5B-E), an inverse relationship existed between extent of the central granular cerebriform zone and level of antibiotic.

The presence of cycloheximide affected development of the aerial mycelia of both strains of *Cladosporium*. Since the response in the two strains was similar, only strain 242 is shown in Fig. 6. On control medium, the colony consisted of a flat, radially grooved mat. Cycloheximide restricted aerial growth so that at 5.0 mg/ml the colony was glabrous. As the concentration of antibiotic was lowered, aerial mycelia became more extensive, and at the lower levels of cycloheximide (0.5 mg or less/ml) the central region of the mycelial mat became cerebriform.

The colonial morphology of one of the two strains of *A. boydii*, i.e., strain 17A, was altered by cycloheximide. On control medium, as illustrated in Fig. 7, the mycelial mat was flat and cottony, whereas on cycloheximide-containing medium, at all concentrations, the colonies were downy and mounded, descending in terraces to a flat periphery.

Finally, cycloheximide changed the morphology of both strains of *Scopulariopsis* radically and in a similar fashion (illustrated in Fig. 8 by strain 326). The mycelial mat on the control medium was flat and ungrooved. In contrast, at all concentrations of antibiotic, the colonies were conical in shape and deeply grooved.

**DISCUSSION**

In Whiffen's early investigations (16, 17) of the action spectrum of cycloheximide upon bac-
teria, yeasts, and zoopathogenic filamentous fungi, the inhibitory activity of the antibiotic against yeasts and bacteria was determined by the tube dilution technique and against filamentous fungi by means of a uniform suspension of spores streaked over the surface of nutrient agar plates containing varying levels of antibiotic. As a result of this and subsequent investigations (2–5, 8) utilizing similar inoculation procedures and qualitative or semiquantitative methods of assessing the activity of cycloheximide, it is now widely assumed that almost all zoopathogens, and in particular dermatophytes, are resistant to the antibiotic, whereas saprophytes and phytopathogens are sensitive to the drug. The results of the present study clearly indicate that this division of the fungi is too simplistic, for the response of an organism to cycloheximide is quite variable and dependent upon a number of factors: concentration of antibiotic, species and strain of microorganism, and duration of exposure to the drug.

Although our data demonstrate that the response to cycloheximide is far more complex than was indicated in earlier studies, the results are in general agreement with those reports which demonstrated the value of this antibiotic in selective isolation media. The addition of cycloheximide at 0.5 mg/ml to a standard nutrient medium would facilitate the isolation of zoopathogens by inhibiting the development of contaminating saprophytes.

However, the efficacy of cycloheximide decreases with the duration of exposure of the con-
Fig. 6. Morphology of Cladosporium sp. strain 242 on control medium (D) and on cycloheximide: (A) 5.0 mg/ml; (B) 1.0 mg/ml; (C) 0.1 mg/ml. Scale = 10 mm.

Fig. 7. Morphology of A. boydii strain 17A on control (left) and 1.0 mg of cycloheximide/ml (right). Scale = 10 mm.
taminants to the antibiotic. While the initial development of both strains of *Cladosporium* was severely inhibited at 0.5 mg of cycloheximide per ml, by the completion of the experiment the percentage of growth equaled or surpassed that of the zoopathogens. Therefore, if a cycloheximide medium is employed, it would seem advisable to accomplish isolation of the zoopathogens within 3 to 5 days after inoculation.

It is somewhat difficult to compare our data with the results of previous studies, but significant differences appear to exist with respect to *A. boydii, Cladosporium,* and *Scopulariopsis.* Georg et al. (5) reported that after 21 days the growth of *A. boydii,* at all concentrations of antibiotic, never exceeded 50% (2+ compared to the development on control; control = 4+) of that found on the control. This contrasts sharply with our data which show that growth at all concentrations of cycloheximide, with the exception of 5.0 mg/ml, was 70 to 80% of that on control. Our results are in close agreement with those reported by Cazin and Decker (2).

With respect to *Cladosporium,* Phillips and Hanel (8) observed that growth was completely inhibited at 0.1 mg of antibiotic/ml, and Georg et al. (5) reported that two of three strains achieved only about one-fourth of the growth found on the control. In our studies, the growth rate of *Cladosporium* was found to increase during the experiment, so that by the completion of the test both strains had attained, at all concentrations of antibiotic, at least 60% of the growth found on the control.

Georg et al. (4) reported an inverse relationship between growth of *Scopulariopsis* and concentration of cycloheximide. On the eighth day after inoculation, they found that the growth of this organism ranged from approximately 75% (3+) at a concentration of 0.1 mg/ml to only 25% (1+) at 1.0 mg/ml. Although we noted a similar relationship, the sensitivity of our strains was much greater.

The increase in the growth rate of the test organisms suggests an induced adjustment or the development of resistance during exposure to cycloheximide. Induced adjustments noted in the literature have dealt primarily with saprophytic yeasts or phytopathogenic molds. For example, Grover and Moore (6) reported a 100-fold increase in resistance of *Sclerotinia laxa* or *S. fruticola* after serially transferring these organisms to progressively higher concentrations of antibiotic. Whiffen (16) noted the rapid development of resistance to cycloheximide in a strain of *Saccharomyces pastorianus*; whereas this organism was originally sensitive to 0.06 mg of antibiotic/ml, a strain was obtained through successive transfers to higher concentrations which was resistant to 4.0 mg/ml. The present report apparently is the first to describe induced resistance to cycloheximide in zoopathogenic fungi.

**LITERATURE CITED**


